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Gallinacins: cysteine-rich antimicrobial peptides of chicken leukocytes

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Abstract

We purified three homologous antimicrobial peptides ('gallinacins') from chicken leukocytes, examined their antimicrobial activity in vitro, and established their primary sequences by a combination of gas phase microsequencing and on-line LC-ESI-MS analysis of endo- and exoprotease peptide digests. The peptides contained 36–39 amino acid residues, were relatively cationic due to their numerous lysine and arginine residues, and each contained 3 intramolecular cystine disulfide bonds. Gallinacins showed primary sequence homology to the recently delineated β-defensin family, heretofore found only in the respiratory epithelial cells and neutrophils of cattle, suggesting that β-defensins originated at least 250 million years ago, before avian and mammalian lineages diverged. The 9 invariant residues (6 cysteines, 2 glycines and 1 proline) common to avian gallinacins and bovine β-defensins are likely to constitute the essential primary structural motif of this ancient family of host-defense peptides.

Key words: Antimicrobial peptide; β-Defensin; Chicken leukocyte; LC-ESI mass spectrometry

1. Introduction

Mammalian phagocytes are equipped with an array of antimicrobial polypeptides which enhance their ability to kill ingested microbes [1,2]. Among these are several well characterized cysteine-rich cationic peptides, including defensins [3,4], β-defensins [5] and protegrins [6]. Defensins have been isolated from polymorphonuclear leukocytes (PMN) of rabbits, humans, rats and guinea pigs; from the alveolar macrophages of rabbits, and from small-intestinal Paneth cells of mice and man (reviewed in [4]). β-defensins, originally isolated from bovine respiratory epithelial cells [7] and bovine PMNs [5], are slightly larger (38–40 aa residues) than classical defensins (29–35 residues), and are not formally homologous to them. Although both β- and classical defensins possess three intramolecular cystine disulfide bonds, neither the spacing of their cysteine residues nor the connectivity of their intramolecular cystine disulfide bonds [8,9] is identical.

Although little is known about the antimicrobial peptides of leukocytes from non-mammalian vertebrates, chicken PMN were shown to contain at least 3 cationic polypeptides that killed *Escherichia coli*, *Serratia marcescens* and *Staphylococcus albus* in vitro [10,11]. Although these antimicrobial molecules were not precisely characterized, they were deemed arginine-rich by histochemical criteria (ammoniacal silver reactivity), and were reported to be stored in large (1–3 μm diameter), rod-shaped dense cytoplasmic granules [11] whose contents discharged into phagosomal vacuoles that formed after chicken PMN ingested bacteria [10]. This report describes the purification and characterization of three cationic, lysine and arginine-containing, cysteine-rich antimicrobial peptides ('gallinacins') from the leukocytes of the domestic chicken, *Gallus gallus*. Remarkably, these peptides proved to be homologous to the recently described bovine β-defensins.

2. Materials and methods

2.1. Chicken peritoneal neutrophils

Sterile peritoneal exudates were harvested from 30 adult Cross Broiler-6 strain chickens 6–8 h after injecting 200 ml of saline with 0.5% starch. Approximately 4.5×10^9 cells, (95% heterophils) were extracted with 40 ml of ice cold 10% HOAc, and the supernatants were concentrated by vacuum centrifugation.

2.2. Purification of gallinacins

The dried extract was dissolved in 5% HOAc and chromatographed on Acrilex P-10 (Reanal, Hungary). Gallinacins emerged slightly after one bed volume, and were purified on a 4.6×250 mm Vydac C-18 RP-HPLC column using a linear gradient of acetonitrile with 0.1% TFA. The first peak (partially purified gallinacin-1) was rechromatographed using 0.13% HFBA for ion pairing. The second major peak (a mixture of Gal-1α, Gal-2 and Gal-3) was purified by preparative continuous acid urea gel electrophoresis (CAU-PAGE) with a Bio-Rad Prep Cell 491 (Bio-Rad, Hercules, CA) as previously described [12], followed by reversed-phase HPLC. Gallinacin 3, which was N-terminally blocked, will not be described further in this report.

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2.3. Antimicrobial testing

Column fractions and final products were tested against *Escherichia coli* ML-35, *Listeria monocytogenes* strain EGD and *Candida albicans* strain 820, by an ultrasensitive radial diffusion technique [13]. The underlay agars contained pH 7.4 buffer (9 mM sodium phosphate, 1 mM sodium citrate), 1% w/v agarose and 0.30 mg/ml of trypticase soy broth powder (BBL, Cockeysville, MD).

2.4. Biochemical analysis

Tricine-SDS-PAGE [14] and AU-PAGE [15] were performed in mini-gel formats (SE 250 unit, Hoefer Scientific, San Francisco, CA). Purified gallinacins were hydrolysed in vacuo in 5.7 N HCl for 36 to 40 h, derivatized with PITC and quantitated as phenylthiocarbamyl derivatives [16]. Half-cystine residues were quantified with performic acid-oxidized peptides and reduced and carboxymethylated (CM-) peptides after desalting by RP-HPLC. Approximately 400 pmol of each CM-gallinacin was microsequenced (ABI 475A sequencer).

2.5. Endoprotease mapping of carboxymethylated (CM) gallinacins

Arg-C digestion of CM-Gal-1 was performed at an enzyme/substrate mass ratio (E/S MR) of 1/50 with 1 nmol of CM-Gal-1 in 55 μl of 0.1 M Tris, 10 mM $CaCl_2$, 5 mM DTT and 0.5 mM EDTA, pH 7.6. Lys-C digestion (E/S MR 1/30) was done with 1.5 nmol each of CM-Gal-1 and CM-Gal-1α in 22 μl of 25 mM Tris buffer (pH 8.5), 1 mM EDTA and 0.5 M urea (pretreated with methylamine). After 16 h at 37°C, the digestions were quenched by adding glacial HOAc, final 25% v/v. The digests were dried, dissolved in 0.1% TFA and purified by RP-HPLC. Amino acid composition of each fragment was determined, and selected fragments were sequenced.

Trypsin digestion (E/S MR 1/50) was performed with 2 nmol of CM-Gal-2 in 20 μl of 0.1 M NH_4HCO_3, pH 8.15, for 4 h at 37°C. Amino acid composition of each purified tryptic fragment was determined and the C-terminal fragment was identified. Approximately 100 pmol of this fragment was subjected to sequence and mass analyses.

2.6. Carboxyl-terminal sequencing

The carboxyl-terminal amino acid of Gal-2 was determined by analyzing residues released by carboxypeptidase-Y. Since our amounts of purified Gal-1 and 1α were limited, 'on-line' mass analysis was used to ascertain their carboxyl-terminal amino acid sequences.

Approximately 200 pmol each of CM-Gal-1 or 1α in 8 μl of 0.1 M NH_4OAc, pH 6.2 was incubated at 37°C for 10 min. Carboxypeptidase-Y was added (E/S MR 1/75), aliquots were removed after 3, 5 and 40 min, and quenched with glacial HOAc (25% v/v final) and frozen for subsequent LC-MS analysis. CM-gal-2 (3 nmol) was digested with carboxypeptidase-Y as above, aliquots were removed at 1.5, 3, 5 and 10 min and the released amino acid(s) were determined as phenylthiocarbamyl derivative(s).

2.7. LC-Electrospray mass spectrometry

Mass spectrometric analyses were performed on a triple quadrupole TSQ-700 mass spectrometer (Finnigan-MAT, San Jose, CA) equipped with an electrospray ion source operating at atmospheric pressure. Mass spectra were recorded in positive ion mode. The microcapillary HPLC system, constructed at the City of Hope (Duarte, CA), consisted of a fused silica column with an inner diameter of 250 μm packed with Vydac 3 μm C18 RP support. A detailed description of the design and operation was reported recently [17,18]. Mass scans were acquired every three seconds in a mass range from 500–2000 and the data collection was monitored using both the base peak (representing the highest intensity per scan) and the reconstructed ion current profile (representing the continuous collection of the total ion current per scan). Spectra were generated by averaging the scans containing the peak, and the mass assignments were made using the Finnigan MAT BIOMASS data reduction software.

3. Results and discussion

3.1. Purification of gallinacins

Fig. 1A illustrates the initial RP-HPLC purification of the low molecular weight peptide mixture recovered after Acrilex P10 chromatography. Gallinacins emerged in two peaks between 36–40% acetonitrile. Gallinacin-1

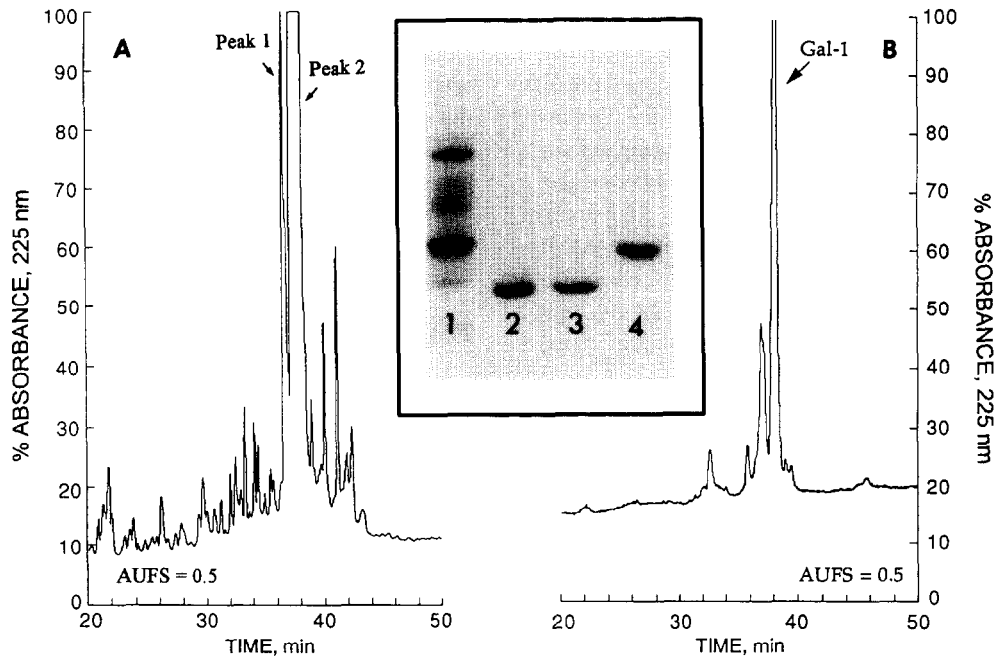

Fig. 1. A: RP-HPLC purification of gallinacins. Pooled, concentrated P-10 fractions were chromatographed on a Vydac C-18 column using a linear, 1% /min gradient of acetonitrile that contained 0.1% TFA. Peak 1 (mainly gallinacin-1) was repurified by RP-HPLC. Peak 2 (a mixture of gallinacins-1α, -2, and -3) was subjected to CAU-PAGE purification. B: Further purification of gallinacin-1. Peak-1 (Fig 1A) was rechromatographed with linear, 0.5% /min gradient of acetonitrile that contained 0.13% HFBA to yield pure gallinacin-1. AUFS signifies absorbance units full scale, and corresponds to 100% absorbance on the ordinate. *Insert:* AU-PAGE of purified gallinacins. Lane 1, ~10 μg of mixture of low molecular weight chicken peptides prior to RP-HPLC purification; Lanes 2–4 contain 1 μg each of purified gallinacin-1α, -1, and -2. The gel was stained with Coomassie blue that contained 15% formaldehyde.

		1		10						20				30		
Gal 1α		GRKSD	C	FRKN	G	F	C	AFLK	CP	YLTLIS	G	K	C	SRFHL-	CC	KRIW
Gal 1		GRKSD	C	FRKS	G	F	C	AFLK	CP	SLTLIS	G	K	C	SRFYL-	CC	KRIW
Gal 2		LF	C	--KG	G	S	C	HFGG	CP	SHLIKV	G	S	C	FGFRS-	CC	KWPWNA
TAP		NPVS	C	VRNK	G	I	C	VPIR	CP	GSMKQI	G	T	C	VGRAVK	CC	RKK
BNBD1		DFAS	C	HTNG	G	I	C	LPNR	CP	GHMIQI	G	I	C	FRPRVK	CC	RSW
BNBD2	V	RNHVT	C	RINR	G	F	C	VPIR	CP	GRTRQI	G	T	C	FGPRIK	CC	RSW
BNBD5	pEVV	RNPQS	C	RWNM	G	V	C	IPIS	CP	GNMRQI	G	T	C	FGPRIK	CC	RSW
BNBD10	pEGV	RSYLS	C	WGNR	G	I	C	LLNR	CP	GRNRQI	G	T	C	LAPRVK	CC	R
BNBD11		GPLS	C	RRNG	G	V	C	IPIR	CP	GPNRQI	G	T	C	FGRPVK	CC	RSW

Fig. 2. Amino acid sequences. The primary structures of gallinacins are shown, numbered according to gallinacin-1α, and aligned with bovine tracheal antimicrobial peptide (TAP) and representitive bovine neutrophil peptides (BNBD). The 9 conserved residues are boxed.

(peak 1) was purified with a slower acetonitrile gradient that contained 0.13% HFBA (Fig. 1B). Gallinacins 1α and -2 and -3 (peak 2) were resolved by sequential CAU-PAGE and RP-HPLC. The purified gallinacins were homogeneous by AU-PAGE (Fig. 1) and silver-stained Tricine-SDS-PAGE (not shown).

3.2. Characterization of gallinacins

Amino acid composition analysis indicated that 6 half-cystine residues were present and that gallinacin-1α and -1 were very similar (data not shown). Automated sequence analysis was carried out on ~ 400 pmols of each carboxymethylated peptide, supplemented and con-

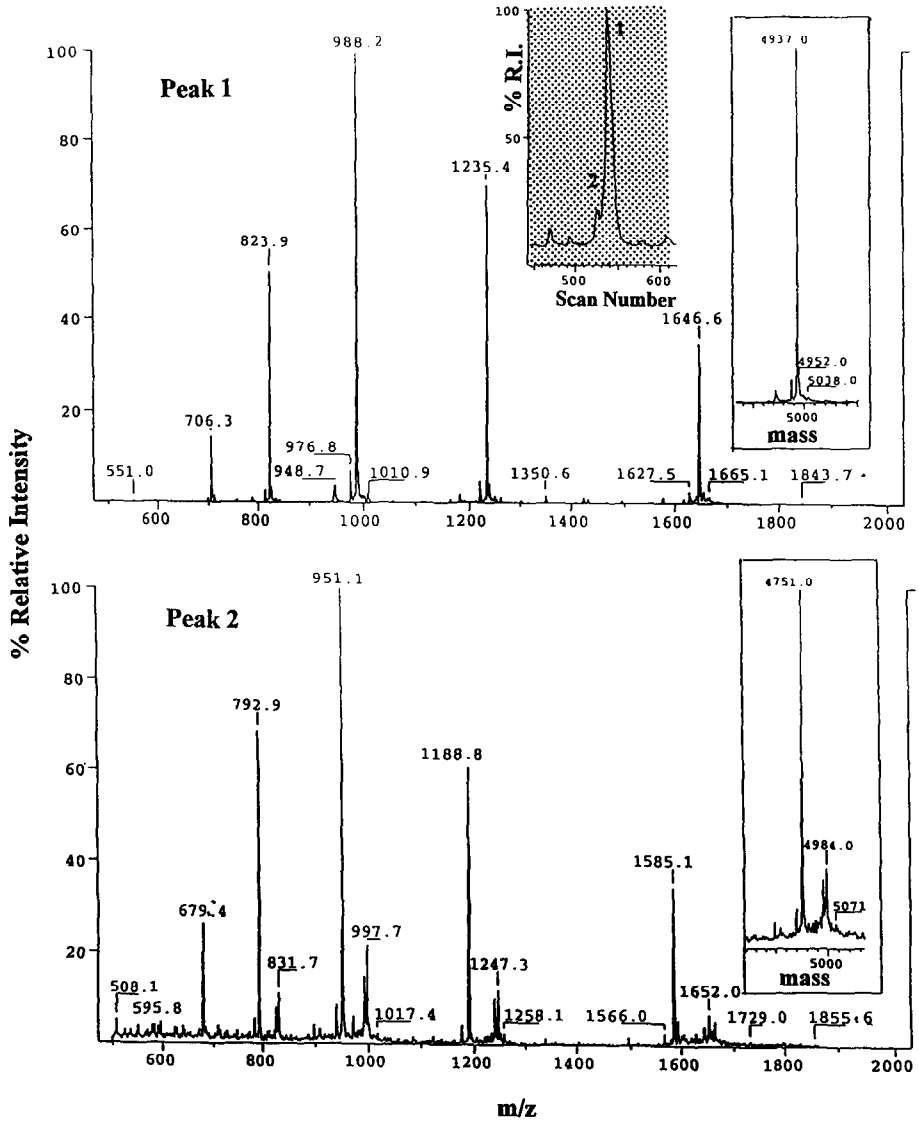

Fig. 3. LC-ESI-MS analyses of CM-Gal-1α digestion mixture Top: the Shaded inset shows the reconstructed ion current profile of 50 pmol of the 3 min carboxypeptidase-Y digest. Peak 1 indicates undigested material, Peak 2 shows the first cleavage product. The mass spectra derived from peak 1 (top) and peak 2 (bottom) are also shown. The corresponding deconvoluted masses are shown in the insets.

firmed by endoprotease and carboxypeptidase-Y digestions. All final data were unambiguous. The greatest challenges involved placement of the carboxyl-terminal residues. Identification of Ala and Asn in Gal-2 was accomplished conventionally by kinetic measurement of amino acid(s) released by carboxypeptidase-Y. The third residue, Trp, was placed by mass measurement of the C-terminal tryptic fragment. Since residues at positions 32 (Trp) and 33 (Pro) had already been established by two independent sequence runs, the mass value of 673.3 obtained for this fragment indicated that another Trp was the only possible fit at position 34. (Note, that position 34 of Gal-2 corresponds to residue 37 of Gal-1α, which was used to establish the numbering scheme shown in Fig. 2). The presence of two tryptophan residues in the Gal-2 sequence was further confirmed (data not shown) by spectrophotometric measurement [19].

We placed the carboxyl-terminal tryptophan in gallinacin-1α and gallinacin-1 by 'on-line' LC-Electrospray-Mass-Spectrometric analysis of carboxypeptidase-Y digestion mixtures. As indicated by the reconstructed ion current profile (Fig. 3, Shaded inset), several peaks were obtained when we subjected the 3 min carboxypeptidase digestion mixture of CM-Gal-1α to LC-MS. The corresponding mass spectra of the major peak (Peak 1) and the peak immediately preceeding it (Peak 2) are also shown in Fig. 3. The deconvoluted average masses for these two peaks (insets) were 4937.0 and 4751.0, respectively. The first value corresponds to the undigested CM-Gal-1α mass value. The second value, 186 mass units less than that of the undigested peptide, is consistent only with tryptophan being the carboxyl-terminal residue, which was also consistent with our spectrophotometric measurements (data not shown).. A similar mass difference was observed with carboxypeptidase digestion studies of CM-Gal-1.

The calculated average mass values of native gallinacin-1α, gallinacin-1 and gallinacin-2, if the 6 half-cystines are linked in three intramolecular disulfide bonds, are 4,581.51, 4,504.43 and 3,915.58. The respective ESI-MS measurement yielded average mass values of 4,582.0, 4,505.0 and 3,916.0, consistent with the expected values.

As shown in Fig. 2, Gal-1α and Gal-1 differed only in three positions, Asn^{10}/Ser^{10}, Tyr^{20}/Ser^{20} and His^{32}/Tyr^{32}. The additional positive charge resulting from the difference at residue 32 is consistent with Gal-1α migrating slightly in advance of Gal-1 in AU-PAGE gels, as noted in Fig. 3. Gal-2 (36 residues) was identical to Gal-1 (39 residues) in 15 of its 36 residues (41.7%), notwithstanding its 3 residue N-terminal truncation and 2 residue C-terminal elongation relative to Gal-1. Gal-2 also differed from Gal-1 and Gal-1α by having only four residues, instead of six, between its first and second cysteines. The overall effect of these truncations was to delete three positively charged residues from the amino terminus of Gal-2, perhaps accounting for its very poor activity, relative to Gal 1 and Gal 1α, against *C. albicans* (vide infra).

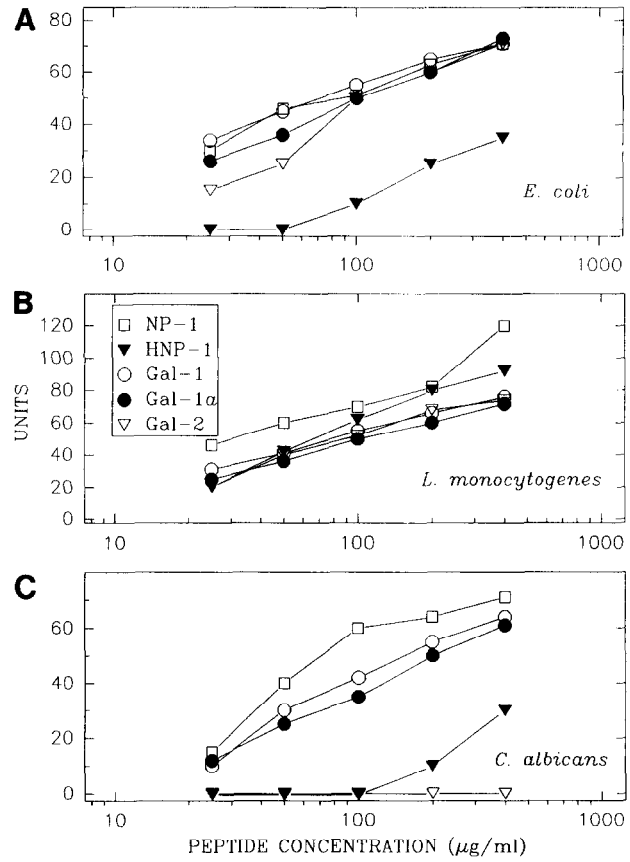

Fig. 4. Antimicrobial activity of purified gallinacins. Purified gallinacins, rabbit defensin NP-1 and human defensin HNP-1 were tested against *E. coli* ML-35p, *L. monocytogenes* EGD and *C. albicans* by radial diffusion assay. Stock concentrations of gallinacins and HNP-1 were measured by absorbance at 280 nm and verified by amino acid analysis. NP-1 concentrations were determined by amino acid analysis only.

When we aligned the gallinacin sequences with those of bovine tracheal antimicrobial peptide ('TAP' [7]) and several bovine neutrophil β-defensins (BNBD, [5]) striking homology was apparent, involving all six conserved cysteine residues as well as three other residues (Gly^{11}, Pro^{19} and Gly^{26}) as shown in Fig. 2. The spacing of the gallinacin cysteine residues was virtually identical to that of β-defensins, but it was quite distinct from that of classical defensins.

3.3. Antimicrobial activity of gallinacins

Gal-1 and 1α were approximately as powerful against *E. coli* ML-35 as was the highly efficacious rabbit neutrophil defensin NP-1, and they were both approximately 10-fold more potent than human defensin HNP-1. Although Gal-2 was slightly less effective, its activity also far exceeded that of HNP-1 (Fig. 4A). Gallinacins 1, 1α and 2 were highly and almost equally effective against *L. monocytogenes*, strain EGD (Fig. 4B). Although Gal-1

and 1α killed *C. albicans* well, Gal-2 was ineffective even when applied at concentrations of 400 mg/ml (Fig. 4C)

It is increasingly evident that mammals use various endogenous antimicrobial peptides for host defense, including cysteine-rich defensins [4], β-defensins [5,7], protegrins [6], bactenecin dodecapeptide [20], eNAPs [21,22], several proline and arginine-rich molecules [23,24], and cecropin-like peptides [25]. Although peptides analogous to mammalian defensins and protegrins have also been found in invertebrates, including insects (e.g. 'insect defensins' [26,27]) and horseshoe crabs (e.g. 'tachyplesins' [28,29]) and similar cysteine-rich antimicrobial peptides are also produced by many plants [30], surprisingly little is known of the evolutionary history and relatedness of these various peptide families.

In their function and primary structure, chicken gallinacins closely resembled β-defensins, recently described antibiotic peptides produced by the respiratory tract epithelial cells [7] and PMN [6] of cattle. Although bovine β-defensins and classical defensins have different disulfide pairing patterns [8,9], the disulfide pairing of gallinacins remains to be established.

All of the 9 invariant residues common to gallinacins and the 14 currently known bovine β-defensins, are important determinants of molecular shape: the six cysteines by forming intramolecular disulfide bonds and Gly^{11}, Pro^{19}, and Gly^{26} by participating in invariant turns [31]. The strict conservation of these nine residues implies that the 'fold' of gallinacins and β-defensins will prove to be very similar. As the likely essential structural elements of the β-defensin, these 9 residues merit designation as the 'β-defensin core-motif'.

The finding of β-defensin-like antimicrobial peptides in chicken indicates that β-defensins are phylogenetically ancient effector molecules that arose before the avian and mammalian lines diverged, over 250 million years ago. Further insights into the evolutionary history of β-defensins (and other components of the molecular armamentarium inherited by man and other mammals) could result from performing analogous biochemical studies to those described here with leukocytes and other tissues from birds and other 'lower' vertebrates.

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